175 Years of Bilirubin Testing: Ready for Point-of-Care?

Natalie Guirguis, Antoine-Xavier Bertrand, Christopher F. Rose, and Simon Matoori*

Bilirubin was first detected in blood in 1847 and since then has become one of the most widely used biomarkers for liver disease. Clinical routine bilirubin testing is performed at the hospital laboratory, and the gold standard colorimetric test is prone to interferences. The absence of a bedside test for bilirubin delays critical clinical decisions for patients with liver disease. This clinical care gap has motivated the development of a new generation of bioengineered point-of-care bilirubin assays. In this Perspective, recently developed bilirubin assays are critically discussed, and their translational potential evaluated.

1. Introduction

Bilirubin was first detected in blood by German physician Rudolf Virchow in 1847.[1] Virchow described morphological similarities of crystals from old hemorrhages and bile pigment crystals, a finding later confirmed by crystallography.[1] Bilirubin is found in the body in two forms: conjugated (direct) and unconjugated (indirect).[2] This metabolite is primarily derived from the hemolysis of senescent erythrocytes, via the breakdown of hemoglobin.[2] Within the reticuloendothelial system, the iron-containing part of the hemoglobin, known as the heme, is oxidized to biliverdin by heme oxygenase, and biliverdin is then reduced to unconjugated bilirubin by biliverdin reductase.[2] The resulting unconjugated water-insoluble bilirubin is then carried in the blood by albumin toward the liver, where it undergoes glucuronidation by UDP-glucuronyltransferase and becomes conjugated water-soluble bilirubin, ready to be excreted through the bile.[2]

Hyperbilirubinemia (i.e., elevated blood bilirubin levels) may be a sign of liver disease or other life-threatening diseases.[2] In general, unconjugated hyperbilirubinemia is a result of accelerated hemolysis, impaired unconjugated bilirubin uptake by the liver, and decreased bilirubin conjugation, whereas conjugated hyperbilirubinemia is due to impaired conjugated bilirubin excretion.[2] Normal total bilirubin levels in blood are less than 17 μmol L^{-1} in adults. In newborns, the cut-off is higher and depends on age, due to their greater production and faster breakdown of red blood cells, thus producing more bilirubin compared to adults. As a result, jaundice (i.e., yellow sclera or skin due to excess bilirubin in the blood) is a very common condition among newborns, affecting more than 60% of them.[3]

Serum bilirubin is a key biomarker in many liver diseases including viral and alcoholic hepatitis, liver cirrhosis, and autoimmune liver disease. Liver disease is one of the leading causes of death and leads to nearly two million deaths per year worldwide.[4] Leading causes include viral infections, chronic alcohol consumption, obesity, autoimmune diseases and genetic predisposition.[4] If left untreated, liver disease may progress to cirrhosis which is associated with serious and potentially lethal complications (e.g., hepatic encephalopathy).[5] It is therefore critical to diagnose and treat patients with liver disease at an early stage. In clinical practice, bilirubin levels are determined for the diagnosis, staging, prognosis, and assessment of treatment success in many acute and chronic liver diseases.

Bilirubin testing at the point-of-care promises to be transformational for the diagnosis and management of acute and chronic liver disease. Regular testing in the out-patient (e.g., at home, doctor's office, home clinic) and in-patient setting (e.g., bedside) would enable more rapid and earlier liver disease diagnosis, staging, and progression, and more frequent assessment of treatment success. Despite these clinical implications, point-of-care testing of bilirubin remains unachieved. Indeed, point-of-care testing has important requirements: capillary blood, small blood volumes, rapid analysis, simplicity, and high storage stability. As the clinical gold standard for bilirubin testing necessitates large equipment and is thus restricted to laboratory use, efforts have been undertaken to engineer a new generation of point-of-care and home bilirubin tests. In this Perspective, we will present selected investigational bilirubin assays and critically discuss their translational potential.

N. Guirguis, A.-X. Bertrand, S. Matoori
Faculté de Pharmacie
Université de Montréal
Montreal, QC H3T 1J4, Canada
E-mail: simon.matoori@umontreal.ca
C. F. Rose
Hepato-Neuro Laboratory
Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM)
Montreal, QC H2X 0A9, Canada
C. F. Rose
Department of Medicine
Université de Montréal
Montreal, QC H3T 1J4, Canada

**ADVANCED
SCIENCE NEWS**

www.advancedsciencenews.com

**ADVANCED
HEALTHCARE
MATERIALS**

www.advhealthmat.de

2. Bilirubin Testing in Clinical Routine

Serum bilirubin testing is widely used in clinical practice for the diagnosis, staging, prognosis, and assessment of individual response to treatment in many diseases of the liver and biliary tract. In most cases, serum bilirubin is a component in a comprehensive clinical and laboratory workup. In this section, bilirubin testing recommended by clinical practice guidelines is discussed.

Acute hepatitis describes an acute state of liver inflammation due to alcohol consumption, viral infection, and autoimmunity. A clinical diagnosis of alcoholic hepatitis is determined in a patient with rapid development or worsening of jaundice and liver-related complications, with total serum bilirubin levels over 51 µmol L^{-1}.[6] In autoimmune hepatitis, total serum bilirubin is an important marker for diagnosis and is determined regularly after treatment initiation to evaluate treatment response.[7] Furthermore, jaundice is part of the diagnostic workup in acute hepatitis due to hepatitis virus B, C and E infection.[8–10]

Liver cirrhosis is the remodeling of the liver into fibrotic tissue. In cirrhosis, total serum bilirubin levels are prognostic of survival after a surgical procedure to reduce ascites (Transjugular intrahepatic portosystemic shunting).[5] This procedure is not recommended if total serum bilirubin levels are higher than 51 µmol L^{-1}.[5] Furthermore, patients with cirrhosis and high serum bilirubin levels are at high risk of developing a first episode of spontaneous bacterial peritonitis.[5] This biomarker is also used in selection of treatment for spontaneous bacterial peritonitis, as intravenous albumin is particularly useful in patients with total serum bilirubin higher than 68 µmol L^{-1} at baseline.[5] Total serum bilirubin is also a factor in the decision for primary prophylaxis for spontaneous bacterial peritonitis in patients, especially in presence of low ascitic fluid protein concentration.[5]

Neonatal jaundice is highly prevalent in newborn babies with ≈60% of term and 80% of preterm babies developing jaundice in the first week of life.[3] Bilirubin cut-off levels depend on age, and guide treatment choice (phototherapy or exchange transfusion).[3] The risk of kernicterus, permanent brain damage due to neurotoxicity of unconjugated bilirubin, is particularly high with bilirubin levels over 340 µmol L^{-1}.[3] However, in case of prolonged jaundice, conjugated bilirubin concentration (cut-off 25 µmol L^{-1}) should be measured to check for serious liver disease.[3] As free (i.e., non-plasma protein-bound) bilirubin distributes into the brain and causes neurotoxicity in newborns, several clinical studies in human neonates found strong correlations of free bilirubin levels in serum with neurological outcomes.[11–13] However, free bilirubin is not commonly assessed in clinical practice because this biomarker is cumbersome to measure and limited to specialized laboratories.[11]

Primary biliary cholangitis is an autoimmune-mediated disease that leads to the destruction of bile ducts.[14] Increased conjugated serum bilirubin is a predictor of poor outcome in these patients, and particularly elevated at late disease stage.[14] It is also used to assess treatment success with the first- and second-line treatments, ursodeoxycholic acid and obeticholic acid, respectively.[14]

Several drugs damage the liver and lead to hepatotoxicity within hours or days. Total serum bilirubin is an important biomarker for acetaminophen-induced liver disease (threshold: 2 x upper limit of normal).[15] While acetaminophen remains the most common hepatotoxic drug, immune checkpoint inhibitors have recently been recognized to induce immune-mediated liver injury.[15] In these patients, total serum bilirubin is used to assess the severity of liver disease, therapy response, and the risk of chronicity.[15]

Bilirubin is also used to assess the risk of mortality from liver failure and thus the need for liver transplantation.[16] Serum bilirubin is part of the model for end-stage liver disease (MELD) score, a widely used predictor of the severity of liver disease used for prioritization of liver transplants, that contains serum creatinine, international normalized ratio of prothrombin time (a marker of blood coagulation), and serum sodium levels.

3. Clinically Used Bilirubin Assays

In clinical practice, the gold standard for determining serum and plasma bilirubin levels is a spectrophotometry test based on a diazo reaction developed by Jendrassik-Grof.[17] In this reaction, sulfanilic acid is co-incubated with sodium nitrite in an acidic medium, which leads to the formation of diazotized sulfanilic acid.[18,19] This molecule reacts with bilirubin to form azobilirubin that absorbs at ≈550 nm. This test has a lower limit of detection of ≈1.5 µmol L^{-1} and a linear range to ≈500 µmol L^{-1} according to one manufacturer (BR411, BR3859, Randox Laboratories, Crumlin, United Kingdom). To discriminate between conjugated and unconjugated bilirubin, caffeine-sodium benzoate is added to the reaction to displace unconjugated bilirubin from albumin and thus release it into solution, where it reacts with diazotized sulfanilic acid.[20,21] The clinical gold standard has several limitations. The test generally corrects for non-specific protein interferences such as immunoglobulins with an averaged plasma protein level that does not take into account inter- and intraindividual differences in plasma protein levels.[22,23] Furthermore, hyperlipidemia (i.e., high blood lipid levels) is a known interference for the bilirubin assay, and very common among liver disease patients due to disturbed lipid metabolism.[22,24,25] Other reported interferences are hemoglobin, which complicates the analysis of hemolyzed samples, and ascorbic acid after vitamin C supplement ingestion.[25–27] The rate of the Jendrassik reaction is also pH-dependent, which necessitates a sufficient buffer capacity to control the pH of the assay solution.[28] The accuracy of this method in clinical laboratories ranged from 91.6% to 106.9%.[29] Furthermore, the median time-to-result for a total serum bilirubin test in neonates was 105 min between blood draw and availability of the result in hospital computer system.[30]

While clinical practice guidelines recommend the spectrophotometric analysis of bilirubin for liver disease diagnosis, there are further disadvantages to this technique, apart from interferences. This test requires a blood draw, coagulation (serum) or the addition of heparin or ethylene amine tetraacetic acid (plasma), and centrifugation, and its analysis through an automated analyzer that determines total and conjugated bilirubin concentrations. However, automated analyzers are bulky, non-portable devices found primarily in hospital laboratories; thus, blood transport by qualified personnel is required to complete the test. This is problematic, as bilirubin is photo-sensitive, making it essential to protect blood samples from exposure to daylight and artificial light during its transport and processing, and entails the risk of artifacts in case of improper storage or delays in sample analyses.[31]
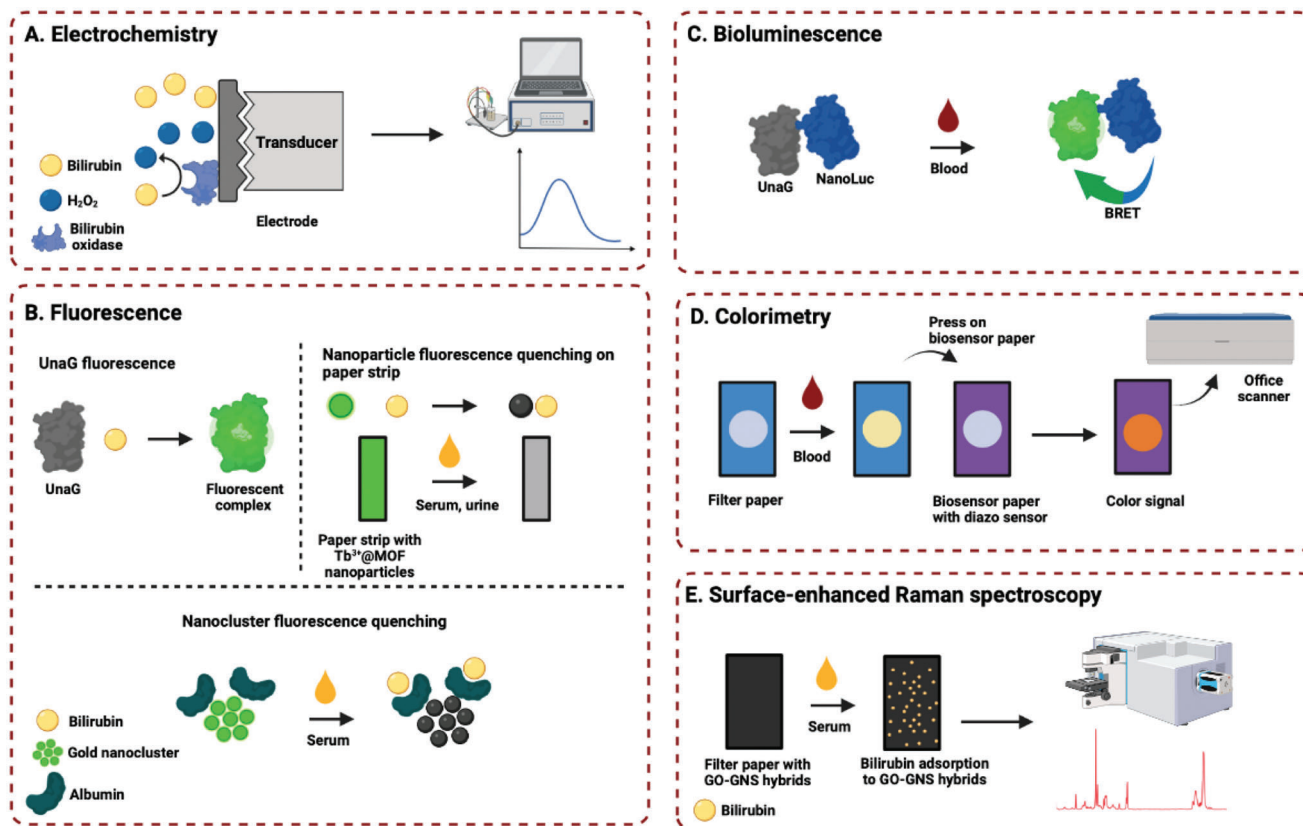
Figure 1. Detection methods of selected investigational bilirubin assays. A) Electrochemistry: an electric current at the electrode surface is induced by direct oxidation of bilirubin or by the hydrogen peroxide generated from the enzymatic oxidation of bilirubin by bilirubin oxidase, and measured by amperometry. B) Fluorescence: the binding of bilirubin to UnaG leads to a fluorescence signal, and bilirubin binding to fluorescent nanoparticles directly or to albumin associated with nanoparticles quenches the fluorescence of the nanomaterials. C) Bioluminescence: bioluminescence resonance energy transfer (BRET) from a luciferase to bilirubin-binding UnaG results in a spectral shift in bioluminescence. D) Colorimetry: Blood is applied to a filter membrane to isolate plasma, which is subsequently pressed on a paper with a bilirubin sensor. E) Surface-enhanced Raman spectroscopy (SERS): bilirubin adsorbs to a filter paper containing graphene oxide plasmonic gold nanostar (GO-GNS) hybrids and induces changes in the SERS spectrum.

Furthermore, this complex process may delay critical treatment decisions such as treatment initiation, treatment choice, or the analysis of the patient's response to treatment (e.g., in cases such as acute liver failure). Overall, these disadvantages motivate the development of new diagnostic bilirubin assays that can determine blood bilirubin levels rapidly and at the bedside.

Other clinically used bilirubin tests include transcutaneous bilirubinometry, smartphone applications and urine strip-based tests. Transcutaneous bilirubinometry is a non-invasive method of measurement for diagnosing jaundice in newborns at the point of care with the Dräger Jaundice Meter JM-103.[32] It is conducted by pressing the meter against the newborn's forehead or sternum, which directs light into the skin and relates the fluorescence by bilirubin to the concentration of total bilirubin in the skin.[32] Transcutaneous bilirubinometry provides results in less than one minute, but lacks the sensitivity and specificity of the standard spectrophotometry test, therefore requiring its use to confirm positive results.[32] Similarly, smartphone applications, such as BiliCam, use a phone's camera along with a color card to assess newborn jaundice with a total bilirubin measurement, and also require the standard test for confirmation.[33] Finally, urine strip-based tests that work by dipping the strip into a urine sam-

ple and visually reading the results according to a color chart can only detect conjugated (water-soluble) bilirubin – the only form that appears in urine – and were reported to yield a high rate of false positive results.[34] Thus, there is a high clinical need for a bilirubin assay that is faster, more sensitive and more specific than the standard spectrophotometry test.

4. Diagnostic Bilirubin Assays under Preclinical Investigation

A variety of investigational diagnostic bilirubin assays have been developed and tested in vitro within bilirubin-spiked human blood, serum, or urine and have thereby shown a preclinical proof-of-concept (**Figure 1**, **Table 1**). While data from clinical tests have not been published yet, the variety of investigational bilirubin assays suggests that new and improved diagnostic tests will soon be available to clinicians and patients with liver disease.

Currently, electrochemical sensors for bilirubin (Figure 1A) are being developed as simple and selective alternatives to spectrophotometry. One sensor was built using screen-printed carbon electrodes (SPE), coated with either electrochemically reduced graphene oxide (Er-GR) or multi-walled carbon

Table 1. Selected investigational bilirubin assays.

Materials	Mechanism of action	Detection method	Linearity range [μmol L^{-1}]	Detection limit [nmol L^{-1}]	Accuracy [%]	Sample volume [μL]	Time-to-result
Screen-printed carbon electrode; graphene oxide; carbon nanotubes[28]	Electrochemical oxidation of bilirubin	Electrochemistry	Er-GR: 0.1-600 MWCNT: 0.5-500	Er-GR: 0.1 ± 0.018 MWCNT: 0.3 ± 0.022	94-106.5	30	5 min
Screen-printed electrode; ceria nanocubes; carbon blacks[35]	Electrochemical oxidation of bilirubin	Electrochemistry	1-100	100	93-102.8	5	20 s
Fluorine doped tin oxide glass plate; graphene oxide nanoparticles; polypyrrole; bilirubin oxidase[36]	Oxidation of bilirubin-by-bilirubin oxidase	Electrochemistry	0.01-500	0.1	96.1-97.5	30	2 s
Gold electrode; zirconia coated silica nanoparticles; chitosan film; bilirubin oxidase[37]	Oxidation of bilirubin-by-bilirubin oxidase	Electrochemistry	0.02-250	0.1	95.6-97	100	2 s
Filter paper; UnaG; β-glucuronidase[38]	UnaG binds bilirubin and becomes fluorescent	Fluorescence	0-34.2	N/A	N/A	1	35 min
Glucose oxidase-peroxidase; ascorbic acid; UnaG[40]	UnaG binds bilirubin and becomes fluorescent	Fluorescence	N/A	N/A	N/A	20	20 s
Tb^{3+}@MOF-808 nanoparticles; filter paper[42]	Fluorescence resonance energy transfer between MOF-808 and bilirubin	Fluorescence	0.4-30	Serum: 23 Urine: 31	95.5-106.7	600	2 min
Human serum albumin-stabilized gold nanoclusters[43]	Quenching of gold nanocluster fluorescence upon binding of bilirubin to albumin	Fluorescence	1-50	248 ± 12	Fluorescence: 98–106 Colorimetry: 97.8-102.2	100	1 min
Conjugate of UnaG and bioluminescent protein NanoLuc[45]	Bioluminescence resonance energy transfer from bioluminescent protein NanoLuc to bilirubin-binding UnaG	Bioluminescence	0-200	N/A	N/A	3	10 min
Caffeine and diazonium-treated filter paper; polysulfone membrane[46]	Colorimetric diazotization of bilirubin which generates azobilirubin	Colorimetry	0-427.5	20 520	N/A	35-50	<10 min
Filter paper; graphene oxide plasmonic gold nanostar (GO-GNS) hybrids[49]	Adsorption of bilirubin to GO-GNS hybrids leading to change in SERS spectrum	Raman spectroscopy	5-75, 125–500	436	82.3-92.6	200	≈22 min

nanotubes (MWCNT).[28] Electrodes treated with nanomaterials are known to yield stronger current responses, as these particles increase surface area and allow for more bilirubin to bind the surface, thus accelerating the electron transport rate.[28] The concentration of free bilirubin was determined by amperometry from an electrochemical workstation with a three electrode system, which uses cyclic voltammetry to monitor the electric current generated by the non-enzymatic electrochemical oxidation of bilirubin to biliverdin and biliverdin to purpurine.[28] Bilirubin was sensed in a reconstituted bilirubin-spiked human serum sample in the linearity range of 0.5–500 μmol L^{-1} using the MWCNT-SPE and in the linearity range of 0.1–600 μmol L^{-1} using the Er-GR-SPE.[37] The graphene coating showed a larger linearity range and better results in terms of sensitivity due to its higher electrical conductivity than carbon nanotubes.[28]

A similar non-enzymatic electrochemical bilirubin sensor was developed based on screen-printed electrodes modified with cerium oxide nanocubes as catalysts for oxidation and carbon blacks as electrical conductors.[35] Here, the oxidation of free bilirubin at the electrode was detected by cyclic voltammetry and differential pulse voltammetry from an electrochemical workstation.[35] An electrical signal was detected in a reconstituted bilirubin-spiked human serum sample in the linearity range of 1–100 μmol L^{-1} and no signal was detected in absence of bilirubin.[35] Potentially interfering molecules were also examined, and it was found that the oxidation peak of bilirubin was well separated from those molecules.[35]

Another sensor was composed of a fluorine doped tin oxide glass plate as the working electrode, coated with graphene for better electrical conductivity, and combined with polypyrrole to further enhance the sensor's electrical conductivity and stability.[36]

Bilirubin oxidase, which oxidizes bilirubin to biliverdin and hydrogen peroxide, was deposited on the modified electrode.[36] Hydrogen peroxide creates the oxidation current that is measured using cyclic voltammetry.[36] Total bilirubin levels were determined in serum samples (stored at −20 °C) from both healthy subjects and patients with jaundice in the linearity range of 0.01–500 µmol L^{-1}.[36] Bilirubin oxidase is a widely used enzyme, and is particularly suitable for electrochemical bilirubin sensors as it oxidizes bilirubin and generates hydrogen peroxide in stoichiometric amounts.[37] However, there are drawbacks to enzyme-based sensors, such as interferences by electroactive substances (e.g., ascorbic acid, uric acid), poor stability, and high costs.[35] A similar bilirubin oxidase-containing sensor was made from a gold electrode with zirconia coated silica nanoparticles immobilized onto its surface using chitosan film.[37] This sensor successfully showed a linear relationship between current and total bilirubin concentrations in reconstituted bilirubin-spiked human serum samples, with a response time of two seconds, but exhibited a less broad linearity range of 0.02–250 µmol L^{-1} than the previous bilirubin oxidase-containing sensor.[37]

Altogether, these electrochemical sensors give rapid responses in serum, and in clinically relevant linear ranges. Thus, they have the potential of becoming small point-of-care devices. They primarily differ in terms of selected materials that modify electrocatalytic properties, electrode stability periods, and costs. Clinical studies in fresh patient blood with comparisons with the clinical gold standard are needed to determine the true potential of these systems in terms of linear range and detection limit in a clinically relevant matrix.

Fluorescence spectroscopy (Figure 1B) is also being investigated as a rapid, inexpensive, and specific means of measuring bilirubin levels. A new generation of portable fluorometers allows for fluorescence measurements at the point-of-care. A protein from Unagi eel muscle was recently discovered to become fluorescent upon binding to bilirubin and named UnaG.[38] UnaG binds selectively to unconjugated bilirubin, and the UnaG-unconjugated bilirubin complex emits at 527 nm.[39] In order to measure total bilirubin concentrations, the conjugated bilirubin needs to be converted to unconjugated bilirubin. To do so, *β*-glucuronidase from *E. coli* was used to hydrolyze conjugated bilirubin to unconjugated bilirubin.[38] Total and unconjugated bilirubin had been quantified in samples containing 1 µL of serum from patients with obstructive jaundice in the linearity range of 0–34.2 µmol L^{-1}, and the results showed good correlation with those obtained using the common bilirubin oxidase method.[38] The use of UnaG also allowed for the detection of total bilirubin in 1–25 µL of urine from patients with liver disease or bile-duct neoplasm, with greater accuracy than with the bilirubin oxidase method, due to its very high affinity and specificity for bilirubin.[38] However, this technique requires a 30 min enzymatic pretreatment of bilirubin with *β*-glucuronidase, as well as blood centrifugation to obtain serum since hemoglobin in blood interferes with the fluorescence of the UnaG-unconjugated bilirubin complex, making rapid bilirubin measurement a challenge.[38] In addition, fluorescent spot tests of serum and urine bilirubin with UnaG were attempted, but they provided inaccurate estimates of total bilirubin.[38]

UnaG was also used to improve a bilirubin detection method based on absorbance spectroscopy.[40] In this colorimetric method, the absorbance of total bilirubin in serum was first measured at 460 nm. A two-step enzymatic reaction was then used to quantify unconjugated bilirubin. In the first reaction, glucose is oxidized by glucose oxidase, which generates hydrogen peroxide. In the second reaction, hydrogen peroxide is used by a peroxidase to oxidize unconjugated bilirubin to a colorless compound.[40] Unconjugated bilirubin levels are calculated by the time required to decrease the initial absorbance by 20%.[40] As the peroxidase is not selective to unconjugated bilirubin, conjugated bilirubin is also oxidized to a colorless compound, which results in an overestimation of unconjugated bilirubin.[40] To improve specificity for unconjugated bilirubin, UnaG was introduced to this method. The binding of UnaG to unconjugated bilirubin provided an initial fluorescence signal, which subsequently decreased due to the oxidation of unconjugated bilirubin by two enzymes.[40] The concentration of unconjugated bilirubin was calculated based on the loss of the fluorescence signal over 20 s.[40] With the UnaG method, free bilirubin was accurately quantified in 20 µL of neonatal serum with high amounts of conjugated bilirubin.[40]

Nanoparticles and nanoclusters have a high potential for bilirubin sensing, as their spectroscopic properties change upon binding to bilirubin or bilirubin-carrying proteins. For a comprehensive discussion of fluorescence nanomaterials-based bilirubin assays, the reader is referred to a recent review on this subject.[41] Another fluorescent bilirubin assay was developed based on terbium-loaded MOF-808 nanoparticles (Tb^{3+}@MOF-808).[42] Metal-organic frameworks (MOFs) are nanocarriers with large surface area and porosity.[32] The fluorescence resonance energy transfer between the nanoparticles and bilirubin results in fluorescence quenching.[42] A linear relationship was demonstrated in serum and urine after 2 min of Tb^{3+}@MOF-808 incubation, with fluorescence spectra measured at an excitation wavelength of 280 nm, in the linearity range of 0.4 to 30 µmol L^{-1}.[42] To translate this assay, the nanoparticles were deposited on papers to generate a portable test strip system. Spraying bilirubin-containing serum and urine onto the test strips resulted in fluorescence quenching in the same bilirubin concentration range.[42] The absence of interference from ions and small organic molecules found in the blood (i.e., ammonium, sodium, potassium, uric acid, glucose, and urea) was also verified.[42] An investigational bilirubin test with a similar mechanism of action was developed based on the interaction between bilirubin and human serum albumin-stabilized gold nanoclusters.[43] The fluorescence of these nanoclusters decreased in a bilirubin concentration-dependent manner in a range of 1 to 50 µmol L^{-1}, and the assay was capable of quantifying bilirubin in human serum samples spiked with bilirubin from 5 to 25 µmol L^{-1}.[43] These nanoclusters further had peroxidase-like activity where bilirubin was oxidized in the presence of the nanoclusters and hydrogen peroxide.[43] The oxidation of bilirubin led to a measurable color loss.[43]

Bioluminescence, a process where light is generated from an enzymatically catalyzed chemical reaction, was also employed to detect bilirubin.[44] The advantage of bioluminescence assays is that a light source for excitation is not required to produce a light signal. A bioluminescence bilirubin assay was developed

**ADVANCED
SCIENCE NEWS**

www.advancedsciencenews.com

**ADVANCED
HEALTHCARE
MATERIALS**

www.advhealthmat.de

based on a protein conjugate composed of a bioluminescent protein, NanoLuc, and UnaG (Figure 1C).[45] In the presence of unconjugated bilirubin, UnaG is a fluorophore and can be excited by bioluminescence resonance energy transfer (BRET) from NanoLuc.[45] Indeed, the NanoLuc-UnaG protein showed a BRET-induced shift in its bioluminescence spectrum in the presence of bilirubin.[45] This assay was functional in whole blood, as this color change was detected in unconjugated bilirubin-spiked mouse blood in a range of 0–200 µmol L^{-1} using a smartphone camera.[45]

Paper-based tests (Figure 1D) are simple, inexpensive, and portable.[46] A colorimetric 3D-printed tape paper sensing device was developed based on the reaction of the gold standard, the colorimetric diazotization method.[46] In this two-part system, a drop of blood is deposited on a polysulfone membrane that separates plasma from whole blood. The plasma diffuses into a caffeine benzoate-containing paper that frees albumin-bound unconjugated bilirubin. A diazonium-indicator-containing second paper is then physically contacted with the first paper to start the chromogenic reaction. The color change was recorded with a conventional office scanner. The study demonstrated a linear relationship between total bilirubin measured with the paper strip sensor and a hospital method, with a linearity range of 0–427.5 µmol L^{-1}.[46]

Surface-enhanced Raman spectroscopy (SERS) is a label-free method for the analysis of analytes at different length scales (small molecules, protein, exosomes) in biological matrices.[47,48] A paper-based SERS bilirubin sensor was developed based on a filter paper, on which graphene oxide plasmonic gold nanostar (GO-GNS) hybrids were deposited (Figure 1E).[49] When GO-GNS hybrids were immersed in bilirubin-spiked buffer and serum for 20 min, bilirubin adsorbed to the GO nanosheets by electrostatic and π-π interactions. After the incubation period, several bilirubin-indicating bands were identified in the SERS spectrum after ≈2 min.[49] The sensor detected bilirubin in bilirubin-spiked fetal bovine serum in the range of 5 to 500 µmol L^{-1}, and showed similar results as the diazo method in the quantification of three bilirubin-spiked serum samples.[49]

In the last decade, a variety of novel bilirubin assays were developed. Among the investigational assays presented in Table 1, the electrochemical, colorimetric and bioluminescence sensors had a broader linearity range than the fluorescence and SERS sensors. Most investigational bilirubin assays exhibited a smaller linear range than the clinical gold standard. Detection limits were lower than the clinical gold standard for electrochemical, fluorescent nanoparticle, and the SERS assays; the colorimetric test exhibited a higher detection limit than the gold standard and the detection limit was not reported for UnaG-based fluorescence and bioluminescence assays. In terms of diagnostic accuracy, the electrochemical and nanomaterials-based tests exhibited comparable accuracy as the clinical gold standard while SERS exhibited a slightly lower accuracy. The studies on the colorimetric and UnaG-based fluorescence and bioluminescence assays did not report accuracy data. The sample volume was higher than the mean fingerstick blood volume of ≈3 µL[50] for most tests except for the bioluminescence and one UnaG-based fluorescence method. The time-to-result was 10 min or less for most investigational assays; two tests, the SERS and one UnaG-based fluorescence method, took over 20 min.

5. Conclusions and Future Developments

Many bilirubin assays with diverse mechanisms of action are under preclinical investigation. Their systems for bilirubin detection mainly apply electrochemical, fluorescent, bioluminescent, colorimetric, and Raman spectroscopy-based methods of analysis. The diversity of these strategies raises hope that clinicians will soon have new and improved bilirubin tests to assess liver disease.

In the last decade, impressive advances were made in bilirubin assays at different length scales and using various detection systems. The discovery of UnaG in 2013,[39] a protein that becomes fluorescent in the presence of bilirubin, spurred the development of several UnaG-based fluorescence and bioluminescence assays.[38,45] Nanomaterials have also greatly inspired the development of new bilirubin assays, where interactions of bilirubin and nanoparticles or nanoclusters led to detectable colorimetric, fluorescence and Raman spectroscopy signals.[42,43,49] In electrochemistry, several potentiometric assays were developed that are either based on the enzymatic oxidation of bilirubin to the electroactive hydrogen peroxide or by directly measuring the potentiometric signal induced by bilirubin.[28,35–37] Moreover, the last decade saw the publication of several specialized reviews that focus on bilirubin assays made of fluorescent nanomaterials[41] and enzymatic sensors,[37,51] as well as comprehensive reviews on bilirubin detection systems.[52,53]

Despite the promising recent advances in bilirubin tests, there are still many challenges to overcome in order to reach the point-of-care. Point-of-care testing has important requirements in terms of blood volume, blood source, assay component stability, cost, assay procedure complexity and required training to use assay, and the dimensions of the detector. Ideally, these tests require low blood volumes of readily accessible capillary blood, have a long shelf-life, are robust, easy-to-use, and inexpensive. The detector should be small, light, inexpensive, and connected to a device to record the measurements (e.g., linked to a smartphone app). Several bottlenecks are hindering the commercialization of investigational point-of-care bilirubin assays. First, blood separation is needed for bilirubin assays that only function in plasma and serum. This may be due to interferences by hemoglobin absorbance or fluorescence (colorimetric and non-near infrared fluorescence assays), or because of interactions of blood components such as erythrocytes and leukocytes with assay components.[54–56] While centrifugation is performed for blood separation in clinical laboratories, options are limited to filtration at the point of care. Despite recent progress, these methods continue to have important limitations including filter clogging, probe dilution, inadequate characterization of filtered plasma composition, and need for pumps in microfluidics-assisted filtration.[57] Another challenge is the stability of the assay components, as high stability at room temperature is desired to provide a long shelf-life. The stability of proteins (e.g., enzymes, bilirubin-sensing proteins), fluorophores, and nanoparticles can be an important limitation. For instance, reported enzyme-modified electrodes required storage at 4 °C in dry conditions.[36] Tape-paper strip sensors similar to the one described may be potential suitable alternatives, as they do not require large laboratory equipment or refrigeration. Furthermore, the development of small and inexpensive detectors is an

important challenge without the use of smartphone cameras. While colorimetric, fluorescent, and bioluminescent signals can be detected with a sensitive smartphone camera, specific detectors are needed for electrochemical systems and Raman spectroscopy. Small portable detectors for electrochemistry are widely used for blood glucose monitoring, and portable Raman spectrometers were developed.[58] Another potential bottleneck in commercialization is manufacturing the bilirubin assay at large scale with highly robust processes and reproducible quality. The price per test is another important factor in commercialization as the price per bilirubin test in clinical laboratory is ≈0.2 USD.[59] The final challenge is a successful validation of the investigational assay in the targeted matrix, the blood of neonates and patients with hepatobiliary disease, in terms of the quality criteria defined by the regulatory authorities including accuracy, precision, linearity, and lower limit of detection and quantification.[60] The complexity and variability of the composition of these matrices is high, as these populations often have strong deviations from the normal range in blood levels of lipids, albumin, electrolytes, hemoglobin, erythrocytes, and leukocytes.[61]

This broad usefulness of bilirubin testing is at the cost of specificity such that bilirubin is often combined with other biomarkers of liver injury (e.g., liver enzymes) and function (albumin). The combination of serum bilirubin and serum albumin is an established marker of post-hepatectomy liver failure and mortality.[62] Moreover, combining serum bilirubin levels with serum lactate, a marker of tissue hypoxia that is metabolized in the liver, was a predictor of mortality in acute liver failure patients.[63] The usefulness of these composite markers highlights the potential of combining point-of-care bilirubin assays with other assays at the point-of-care to allow for a more complete analysis of liver status and higher specificity.

We predict that advances in blood bilirubin assays will provide rapid, specific, and highly sensitive point-of-care options in the coming decade. These bilirubin tests promise to accelerate diagnosis and critical treatment decisions in liver disease and have the potential to revolutionize liver disease testing at the point-of-care if combined with other biomarkers of liver disease.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

[1] P. J. Bosma, *J Hepatol* **2003**, *38*, 107.

[2] G. R. Gourley, R. A. Arend, *Lancet* **1986**, *327*, 644.

[3] J. Rennie, S. Burman-Roy, M. S. Murphy, *BMJ* **2010**, *340*, c2409.

[4] J. Thompson, N. Jones, A. Al-Khafaji, S. Malik, D. Reich, S. Munoz, R. MacNicholas, T. Hassanein, L. Teperman, L. Stein, Andrés Duarte-Rojo, Raza Malik, Talal Adhami, Sumeet Asrani, Nikunj Shah, Paul Gaglio, Anupama Duddempudi, Brian Borg, Rajiv Jalan, Robert Brown, Heather Patton, Rohit Satoskar, Simona Rossi, Amay Parikh, Ahmed ElSharkawy, Mantry, Parvez, Linda Sher, David Wolf, Marquis Hart, Charles Landis, et al., *Liver Transplant.* **2018**, *24*, 380.

[5] P. Angeli, M. Bernardi, C. Villanueva, C. Francoz, R. P. Mookerjee, J. Trebicka, A. Krag, W. Laleman, P. Gines, *J Hepatol* **2018**, *69*, 406.

[6] A. K. Singal, R. Bataller, J. Ahn, P. S. Kamath, V. H. Shah, *Am. J. Gastroenterol.* **2018**, *113*, 175.

[7] M. P. Manns, A. J. Czaja, J. D. Gorham, E. L. Krawitt, G. Mieli-Vergani, D. Vergani, J. M. Vierling, *Hepatology* **2010**, *51*, 2193.

[8] D. Mutimer, A. Aghemo, H. Diepolder, F. Negro, G. Robaeys, S. Ryder, F. Zoulim, M. Peck, A. Craxi, M. Fried, et al., *J Hepatol* **2014**, *60*, 392.

[9] H. R. Dalton, N. Kamar, S. A. Baylis, D. Moradpour, H. Wedemeyer, F. Negro, *J Hepatol* **2018**, *68*, 1256.

[10] N. A. Terrault, A. S. F. Lok, B. J. McMahon, K. M. Chang, J. P. Hwang, M. M. Jonas, R. S. Brown, N. H. Bzowej, J. B. Wong, *Hepatology* **2018**, *67*, 1560.

[11] S. B. Amin, A. A. Lamola, *Semin Perinatol* **2011**, *35*, 134.

[12] W. Oh, D. K. Stevenson, J. E. Tyson, B. H. Morris, C. E. Ahlfors, G. J. Bender, R. J. Wong, R. Perritt, B. R. Vohr, K. P. Van Meurs, H. J. Vreman, A. Das, D. L. Phelps, T. Michael O'Shea, R. D. Higgins, *Acta Paediatr* **2010**, *99*, 673.

[13] C. E. Ahlfors, S. B. Amin, A. E. Parker, *J. Perinatol. 2009 294* **2009**, *29*, 305.

[14] G. M. Hirschfield, U. Beuers, C. Corpechot, P. Invernizzi, D. Jones, M. Marzioni, C. Schramm, *J Hepatol* **2017**, *67*, 145.

[15] R. J. Andrade, G. P. Aithal, E. S. Björnsson, N. Kaplowitz, G. A. Kullak-Ublick, D. Larrey, T. H. Karlsen, *J Hepatol* **2019**, *70*, 1222.

[16] P. Martin, A. Dimartini, S. Feng, R. Brown, M. Fallon, *Hepatology* **2014**, *59*, 1144.

[17] C. V. Hulzebos, L. Vitek, C. D. Coda Zabetta, A. Dvořák, P. Schenk, E. A. E. van der Hagen, C. Cobbaert, C. Tiribelli, *Pediatr. Res. 2021 902* **2021**, *90*, 277.

[18] M. I. Walters, H. W. Gerarde, *Microchem. J.* **1970**, *15*, 231.

[19] R. N. Rand, A. Di Pasqua, *Clin. Chem.* **1962**, *8*, 570.

[20] F. C. Pearlman, R. T. Y. Lee, *Clin. Chem.* **1974**, *20*, 447.

[21] P. Nagaraja, K. Avinash, A. Shivakumar, R. Dinesh, A. K. Shrestha, *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2010**, *77*, 782.

[22] G. Dimeski, *Clin. Biochem. Rev.* **2008**, *29*, S43.

[23] A. Nauti, A. Barassi, G. Merlini, G. V. M. D'Eril, *Clin. Chem.* **2005**, *51*, 1076.

[24] A. Sorokin, J. L. Brown, P. D. Thompson, *Atherosclerosis* **2007**, *194*, 293.

[25] J. Z. Ji, Q. H. Meng, *Clin. Chim. Acta* **2011**, *412*, 1550.

[26] B. C. Shull, H. Lees, P. K. Li3, *Clin. Chem.* **1980**, *26*, 26.

[27] F. Martinello, E. L. da Silva, *Clin. Biochem.* **2006**, *39*, 396.

[28] M. Thangamuthu, W. E. Gabriel, C. Santschi, O. J. F. Martin, *Sensors* **2018**, *18*, 800.

[29] S. F. Lo, *JAMA Pediatr* **2016**, *170*, 529.

[30] N. Y. Boo, Y. F. Chang, Y. X. Leong, Z. Y. Tok, L. C. Hooi, S. C. Chee, Z. A. Latif, *Pediatr Res* **2019**, *86*, 216.

[31] A. G. Sofronescu, T. Loebs, Y. Zhu, *Clin. Chim. Acta* **2012**, *413*, 463.

[32] C. I. Okwundu, O. A. Uthman, G. Suresh, J. Smith, C. S. Wiysonge, V. K. Bhutani, *Cochrane Database Syst Rev* **2017**, *2017*.

[33] J. A. Taylor, J. W. Stout, L. De Greef, M. Goel, S. Patel, E. K. Chung, A. Koduri, S. McMahon, J. Dickerson, E. A. Simpson, et al., *Pediatrics* **2017**, *140*, e20170312.

[34] K. F. Foley, J. Wasserman, *Lab Med* **2014**, *45*, 59.

[35] Z. J. Lu, Y. Cheng, Y. Zhang, X. Wang, P. Xu, H. Yu, X. Li, *Sens Actuators B Chem* **2021**, *329*, 129224.

[36] N. Chauhan, R. Rawal, V. Hooda, U. Jain, *RSC Adv.* **2016**, *6*, 63624.

[37] B. Batra, S. Lata, J. S. R. Sunny, C. S. Pundir, *Biosens. Bioelectron.* **2013**, *44*, 64.

[38] T. T. Tai, Y. Adachi, S. Taketani, *Lab Med* **2022**, *53*, 6.

[39] A. Kumagai, R. Ando, H. Miyatake, P. Greimel, T. Kobayashi, Y. Hirabayashi, T. Shimogori, A. Miyawaki, *Cell* **2013**, *153*, 1602.

[40] S. Iwatani, K. Yamana, H. Nakamura, K. Nishida, T. Morisawa, M. Mizobuchi, K. Osawa, K. Iijima, I. Morioka, *Int. J. Mol. Sci.* **2020**, *21*, 6778.

[41] Y. Guo, C. Wei, R. Wang, Y. Xiang, *Anal. Biochem.* **2023**, *666*, 115078.

[42] K. Yi, H. Li, X. Zhang, L. Zhang, *Inorg. Chem.* **2021**, *60*, 3172.

[43] M. Santhosh, S. R. Chinnadayyala, A. Kakoti, P. Goswami, *Biosens. Bioelectron.* **2014**, *59*, 370.

[44] Z. Xia, J. Rao, *Curr. Opin. Biotechnol.* **2009**, *20*, 37.

[45] Y. Itoh, M. Hattori, T. Wazawa, Y. Arai, T. Nagai, *ACS Sens.* **2021**, *6*, 889.

[46] W. Tan, L. Zhang, J. C. G. Doery, W. Shen, *Lab Chip* **2020**, *20*, 394.

[47] S. Laing, L. E. Jamieson, K. Faulds, D. Graham, *Nat. Rev. Chem. 2017* **2017**, *1*, 0060.

[48] T. J. Moore, A. S. Moody, T. D. Payne, G. M. Sarabia, A. R. Daniel, B. Sharma, *Biosensors* **2018**, *8*, 46.

[49] X. Pan, L. Li, H. Lin, J. Tan, H. Wang, M. Liao, C. Chen, B. Shan, Y. Chen, M. Li, *Biosens. Bioelectron.* **2019**, *145*, 111713.

[50] M. Grady, M. Pineau, M. K. Pynes, L. B. Katz, B. Ginsberg, *J Diabetes Sci Technol* **2014**, *8*, 691.

[51] V. Hooda, A. Gahlaut, A. Gothwal, V. Hooda, *Biotechnol. Lett.* **2017**, *39*, 1453.

[52] L. Ngashangva, V. Bachu, P. Goswami, *J. Pharm. Biomed. Anal.* **2019**, *162*, 272.

[53] V. Narwal, B. Batra, V. Kalra, R. Jalandra, J. Ahlawat, R. Hooda, M. Sharma, J. S. Rana, *Sens. Bio-Sensing Res.* **2021**, *33*, 100436.

[54] S. Matoori, D. J. Mooney, *Small* **2020**, *16*, 2000369.

[55] S. Matoori, D. J. Mooney, *Biomaterials* **2022**, *283*, 121475.

[56] Y. Tian, Z. Tian, Y. Dong, X. Wang, L. Zhan, *RSC Adv.* **2021**, *11*, 6958.

[57] W. S. Mielczarek, E. A. Obaje, T. T. Bachmann, M. Kersaudy-Kerhoas, *Lab Chip* **2016**, *16*, 3441.

[58] J. Sun, L. Gong, W. Wang, Z. Gong, D. Wang, M. Fan, *Luminescence* **2020**, *35*, 808.

[59] M. Salinas, M. López-Garrigós, J. Lugo, M. Gutiérrez, L. Flors, C. Leiva-Salinas, *J Clin Pathol* **2012**, *65*, 928.

[60] A. Spyrogianni, C. Gourmel, L. Hofmann, J. Marbach, J. C. Leroux, *Sci. Reports 2021 111* **2021**, *11*, 22032.

[61] S. Matoori, J. M. Froehlich, S. Breitenstein, V. Pozdniakova, C. Reischauer, O. Kolokythas, D. M. Koh, A. Gutzeit, *Eur Radiol* **2019**, *29*, 5813.

[62] A. M. Fagenson, E. M. Gleeson, H. A. Pitt, K. N. Lau, *J Am Coll Surg* **2020**, *230*, 637.

[63] J. Hadem, P. Stiefel, M. J. Bahr, H. L. Tillmann, K. Rifai, J. Klempnauer, H. Wedemeyer, M. P. Manns, A. S. Schneider, *Clin. Gastroenterol. Hepatol.* **2008**, *6*, 339.